

Effects of Changes in Calmodulin Levels on Cell Proliferation

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Calmodulin (CaM) is one of several proteins regulated in a cell cycle-dependent manner. CaM is synthesized at the G₁/S boundary and has been implicated in the regulation of cell cycle progression. To elucidate the role of calmodulin in cell cycle control, clonal mouse C127 cell lines transformed with one of four different bovine papilloma virus (BPV)-based vectors were studied. These vectors express *a*) a chicken CaM gene regulated by its own promoter (CM cells), *b*) the chicken CaM gene regulated by the inducible human metallothionein-IIa promoter (MCM cells), *c*) CaM antisense RNA using the Zn²⁺ inducible mouse metallothionein-I (mMT-I) promoter (AS cells), or *d*) a rat parvalbumin gene using the chicken CaM promoter (PV cells). C127 cells transformed by BPV-1 alone (BPV cells) are used as a control in each case. Previous studies showed that a 4-fold increase in CaM levels in CM cells shortened the cell cycle by reducing the length of the G₁ period. Expression of parvalbumin in PV cells has no effect on cell cycle length, suggesting that increased CaM, and not simply increased Ca²⁺-binding protein, accelerates proliferation. Zn²⁺-induced expression of the chicken CaM gene in MCM cells increased the rate of proliferation, while Zn²⁺-induced expression of high levels of CaM anti-sense RNA stops proliferation at Zn²⁺ levels that do not affect the growth of BPV cells. In CM cells increased CaM affects cell cycle-dependent level of mRNAs for tubulin, vimentin, and *c-myc* relative to the levels in BPV cells. Tubulin mRNA levels are decreased 2-fold in CM cells due to decreased tubulin mRNA half-life. Vimentin and *c-myc* mRNAs that have been previously shown to respond to mitogenic stimulation are increased in CM cells. We also observe that *c-myc* mRNA increases 2- to 3-fold in both CM and BPV cells as they progress from mitosis to G₁. This is the first demonstration of regulation of *c-myc* mRNA over the course of the cell cycle in exponentially growing cells. Previous studies have shown that CaM is required for quiescent cells to reenter the cell cycle. Quiescent CM cells begin DNA synthesis 2.5 hr earlier than do BPV or PV cells after mitogenic stimulation, indicating that CaM levels have a direct effect on the rate of G₀-S transit. We are currently examining the effect of increased (using CM cells) or decreased CaM levels (via anti-sense RNA induction) on cell cycle-dependent gene expression during cell cycle reentry in response to mitogens.

Introduction

Calmodulin, the ubiquitous Ca²⁺-binding protein, interacts with a large number of intracellular targets and mediates many of the Ca²⁺-dependent functions in nonmuscle cells (1). Calmodulins have been found in all eukaryotes from yeast to mammals (2-5) and show a remarkable conservation of the primary amino acid sequence. Calmodulin-dependent enzymes are involved in a vast array of cellular processes including cyclin nucleotide and glycogen metabolism, secretion, and cell motility. Calmodulin is also thought to be involved in the regulation of cell growth and proliferation. The transformation of cells by chemical carcinogens or oncogenic viruses is usually

accompanied by an increase in the cellular concentration of CaM (6-11). Previous studies with Chinese hamster ovary (CHO) cells have shown that CaM antagonists reversibly block cell cycle progression both in quiescent cells mitogenically stimulated to reenter the cell cycle and in exponentially growing cells (12,13). In addition, CaM and its mRNA are both regulated, although not coordinately, during the cell cycle in exponentially growing CHO cells (12,14; Rasmussen and Means, unpublished observations).

In CHO cells CaM levels double abruptly at the G₁/S boundary of the cell cycle, and this increase is strongly correlated with the entry of cells into S phase (12,13). Together, these observations provided convincing but correlative evidence that CaM levels influenced cell cycle progression, and hence the rate of proliferation.

The present studies were designed to address whether or not a direct relationship exists between CaM levels and cell proliferation. Our approach to more clearly define the CaM role in regulating cell

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proliferation has been to construct a set of bovine papilloma-virus based (BPV-based) eukaryotic expression vectors to elevate or decrease intracellular CaM levels.

Vector Construction and Isolation of Cell Lines

A series of five expression vectors have been constructed and used to stably transform mouse C127 cells (15). Both the vectors and procedures used have been previously described (16). We selected BPV as an expression vector since it is maintained as a stable episome in mouse C127 cells. The result is that the gene of interest is maintained in a consistent chromatin environment. Further, the lack of random integration into the host genome precludes the possibility of altering or disrupting the expression of a gene required for normal cell function. The five vectors used are listed below. The name in brackets is the designation given to isolated cell lines transformed by each expression vector.

(A) pdBPV-1 (BPV Cells)

The vector pdBPV-1 contains the entire BPV-1 genome and sequences from the pBR322 derivative pML2 to allow propagation in *E. coli* (15). This vector serves as a control for the effect of transformation on C127 cells by BPV alone.

(B) BPV-CM (CM Cells)

The vector BPV-CM contains the 69% transforming region of BPV-1 and a chicken CaM whose transcription is regulated by the chicken CaM promoter. Cells transformed by BPV-CM have CaM mRNA levels 20 to 50 times normal and CaM levels which are constitutively 5-fold higher than pdBPV-1 transformed controls (17).

(C) BPV-MCM (MCM Cells)

The vector BPV-MCM contains the 69% transforming region of BPV, and the chicken CaM gene present in BPV-CM but in this instance transcription of the CaM gene is regulated by the inducible human metallothionein-IIa promoter (hMT-IIa), obtained from M. Karin (18). The hMT-IIa promoter was selected since it is regulated by divalent cations (e.g., Zn^{2+}) and glucocorticoids, thus allowing CaM mRNA and protein levels to be elevated in an inducible manner.

(D) BPV-CaMAS (AS Cells)

The vector BPV-CaMAS contains the 69% transforming region of BPV-1 and a 1.0 kb portion of the chicken CaM cDNA (4), including the entire coding sequence, placed in inverted orientation 3' to the

inducible mouse metallothionein-I (mMT-1) promoter (19). This vector allows the inducible production of CaM anti-sense RNA in a stable cell line.

(E) BPV-CaMPV (PV Cells)

In assessing the role of CaM in cell function, one must distinguish between effects due to CaM activity and those which may be attributable to increased levels of the Ca^{2+} -binding protein within the cell. In order to distinguish between these two alternatives, we have produced a vector which expresses a rat parvalbumin cDNA (20) regulated by the chicken CaM promoter. Parvalbumin binds Ca^{2+} with high affinity, but cannot substitute for CaM relative to any of the known activities of the latter protein (21). Further, the use of the CaM promoter to drive the transcription of the parvalbumin gene allows determination of whether or not the cell cycle-dependent expression of CaM mRNA is due to transcriptional or post-transcriptional mechanisms.

Results and Discussion

Clonal cell lines transformed with each of the five vectors described above have been isolated and characterized in detail. Since we were interested in ascertaining whether or not CaM played a direct role in cell proliferation, the effects of increased CaM on growth rate were studied. The growth characteristics of BPV, CM, and PV cells are summarized in Table 1. The results indicate that the increased levels of CaM present in CM cells results in a shortened cell cycle relative to the BPV cell line. Detailed analysis has shown this attributable solely to a reduction in the length of the G_1 period (17). In contrast, expression of parvalbumin in PV cells did not result in a shortened cell cycle. Thus, the effects of increased CaM on cell growth are specific for CaM and are not a result of simply increasing the intracellular Ca^{2+} -binding protein concentration.

In contrast, both PV and CM cells grow to a higher saturation density than do control cells. This suggests that transition from cycling to quiescence may depend on the total intracellular Ca^{2+} -binding capacity and is not specifically related to an increase in CaM levels.

Most of the conclusions regarding the involvement of CaM in regulating cell proliferation has been the result of previous studies that examined the effects of

Table 1. Growth characteristics of transformed cell lines.

Cell line	CaM levels, ng/ 10^6 cells	Cell cycle length, hr	Sat. density, cells/dish
BPV-1	410 ng	14.7 hr	2.3×10^6
PV-7 ^a	419 ng	15.5 hr	4.0×10^6
CM-1	1810 ng	12.7 hr	4.2×10^6

^aPV-7 cells express PV at a concentration of 700 ng/ 10^6 cells. There is no detectable parvalbumin mRNA or protein in BPV-1 or CM-1 cells.

anti-CaM drugs [e.g., naphthalenesulfonamides (22)] on cell proliferation. While these drugs do inhibit CaM activity *in vitro*, their specificity in cells is not absolute, and these agents can also interact with alternative intracellular targets such as protein kinase C.

A more specific method of lowering the effective concentration of CaM would be to prevent its synthesis by the production of a CaM anti-sense RNA complementary to the endogenous CaM mRNA. We have constructed a BPV-based vector (BPV-CaMAS) that contains a chicken CaM cDNA (including 1 kb of sequence beginning with the initiator ATG codon) placed in an inverted orientation 3' to the mouse metallothionein-I promoter. This promoter responds acutely to levels of ZnSO_4 that are not inhibitory to normal cell growth (23).

We have isolated and characterized two independent cell lines (AS-8 and AS-12) which stably maintain the BPV-CaMAS vector and produce high levels of CaM anti-sense RNA when cultured in the presence of $80 \mu\text{M}$ ZnSO_4 . Anti-sense RNA levels increase for 6 to 8 hr following the addition of Zn^{2+} and then decline. This transient effect is characteristic of mMT-I promoter response to induction with Zn^{2+} (23). Concomitant with the appearance of CaM anti-sense RNA, there is a rapid decrease in intracellular CaM levels from 420 ng to 300 ng/ 10^6 cells, a value nearly that which is present in G_1 (250 ng/ 10^6 cells). Between 8 to 12 hr after the addition of Zn^{2+} , CaM levels return to normal as anti-sense RNA levels decline.

The effect of reduced CaM levels on the proliferation of exponentially growing AS cells is striking. From 2 hr until 8 hr after the addition of Zn^{2+} to the culture medium, there is no increase in the number of cells expressing CaM anti-sense RNA. In contrast, an addition of the same concentration of Zn^{2+} to BPV-1 control cells has no effect on proliferation. After 8 hr, the cell number begins to increase again, and proliferation resumes at a rate similar to that of control cells.

In contrast to AS cells, cells transformed with the BPV-MCM vector transiently increase CaM levels 2-fold in response to $80 \mu\text{M}$ ZnSO_4 . The transient elevation of CaM levels results in an increased rate of growth of MCM cells from 2 to 12 hr following an addition of inducer. Thus, a transient increase of CaM levels in MCM cells affects cell proliferation in a manner analogous but opposite of that observed when CaM levels are transiently decreased in AS cells. The observation that cell proliferation is affected immediately on transient alteration of CaM levels suggests two possibilities. First, changes in CaM levels may exert a general effect on cell cycle progression in all cells, regardless of cell cycle position. Alternatively, CaM may act through regulation of a specific event late in the cell cycle that is required for completion of the cell cycle. We are currently examining the cell cycle kinetics during induction of MCM and AS cells in order to differentiate between these alternative modes.

Since constitutive elevation of CaM levels (as occurs in CM cells) results in a shortening of the cell cycle, we were interested in determining if this was accompanied by changes in the levels of mRNAs normally regulated in a cell cycle-dependent manner. Cytoplasmic RNA was isolated from mitotically synchronized cells (24) at various times after mitosis, and the levels of several different mRNAs were quantitated by hybridization (16) to radiolabeled cDNA probes.

Two genes, glyceraldehyde phosphate dehydrogenase and histone H4, were examined since the first has been reported not to change over a variety of growth conditions, and the second provides an internal control for normal progression through the cell cycle, since the mRNA maximally accumulates in the S phase (25). No significant change in cell cycle-dependent levels of either mRNA was observed between BPV and CM cells for either glyceraldehyde phosphate GAPDH or histone-H4 mRNAs. Thus, elevated CaM in CM cells does not cause general changes in mRNA levels during the cell cycle.

However, several mRNAs did demonstrate changes in levels during the cell cycle in CM versus BPV cells. β -actin mRNA was elevated 30% in CM relative to BPV cells. Previous studies have shown that actin mRNA levels are elevated to more rapidly proliferating cells. In contrast, β -tubulin mRNA is decreased nearly 2-fold in CM cells. Measurements of mRNA half-life in BPV and CM cells show that β -tubulin mRNA has a 30% shorter half-life in CM cells, while the half-lives for β -actin and CaM are unchanged. Previous studies both *in vivo* and *in vitro* have demonstrated that CaM can cause Ca^{2+} -dependent depolymerization of microtubules and that increased levels of tubulin dimer cause a decrease in β -tubulin mRNA due to a decrease in mRNA stability. The observation that β -tubulin mRNA is decreased due to a decreased stability in CM cells is consistent with these studies.

Both vimentin and *c-myc* mRNAs have been reported to be regulated in a cell cycle-dependent manner. The vimentin mRNA is normally expressed in G_1 (28) and is increased 50% in CM cells relative to BPV cells. These data might be expected since the G_1 period in CM cells is more rapid than in BPV cells. On the other hand, *c-myc* mRNA was elevated 2-fold in CM cells, compared to BPV cells at all phases of the cell cycle. In addition *c-myc* mRNA was found to increase 2- to 3-fold as cells from either clone progressed from M to G_1 . Previous studies examining *c-myc* mRNA levels have not observed this change, as these studies did not use mitotic selection as a synchronization method and thus were not able to examine changes in mRNA levels prior to mid- G_1 .

Calmodulin has also been shown to be important in the reentry of quiescent cells into the proliferative cycle in response to mitogen. Preliminary studies indicate that CM cells reenter the cell cycle more rapidly than do BPV cells. Since the metallothionein promoters are inducible under any growth conditions, we

are currently examining the effect of induced increases (in MCM cells) or decreases (in AS cells) on the kinetics of cell cycle reentry, and the effects of changes in CaM levels on mRNA levels of genes known to be induced when quiescent cultures are stimulated to reenter the cell cycle (e.g., *c-myc*, *c-fos*, vimentin, actin).

Summary

Calmodulin levels in cells have been increased or decreased by the use of BPV-based expression vectors. Our results have demonstrated a direct relationship between intracellular CaM concentration and cell proliferation. The constitutive elevation of CaM, as occurs in CM cells, exerts effects primarily on the length of the G_1 period, while transient increase (as in MCM cells) or decrease (as in AS cells) of CaM levels may affect proliferation by acting at multiple points within the cell cycle. The cell lines, which we now have, allow us to begin analysis of the intracellular targets through which CaM regulates cell growth.

The authors would like to thank Elizabeth MacDougall and Charles Mena for their excellent technical assistance and Lisa Gamble for the preparation of the manuscript. This work is supported by an American Cancer Society grant, BC-326M to A.R.M.

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